Characterizing a Peptide Motif for the Targeting of Trm1-II to the Inner Nuclear Membrane

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with research distinction* in Molecular Genetics in the undergraduate colleges of The Ohio State University

by

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Abstract

The nucleus of a cell is separated from the cytoplasm by the nuclear envelope, which consists of an inner and outer membrane. The inner nuclear membrane (INM) is home to many proteins. Genetic disorders referred to as "laminopathies", which include cardiac and skeletal myopathies, partial lipodystrophy, peripheral neuropathy, and severe premature aging, are caused by failure to correctly localize proteins to the inner nuclear membrane (INM). To study the process by which peripheral proteins arrive at and are maintained at the INM, I have employed a yeast model system using TRM1-II as a reporter. In yeast, Trm1-II, a tRNA methyltransferase, is peripherally associated with the INM. Previous studies determined that the Trm1 amino acid region 130-151 is necessary and sufficient for INM targeting (Lai et al., 2009). This is the first peptide motif discovered that has INM targeting activity. In order to determine which amino acids within the motif are important for INM targeting, I performed a random mutagenesis screen. The results suggest that structural features of this motif are responsible for the targeting of Trm1-II to the INM. Structural analysis of archeal Trm1 revealed that an α-helix is formed by amino acids 145-153. This α-helix is amphipathic (ie. one side of the helix is comprised of polar residues, while the other is comprised of nonpolar residues). Since amino acid changes that affect the amphipathic property of the α-helix resulted in the mislocalization of Trm1-II to the nucleoplasm, I proposed that the amphipathic property of the helix is important for the INM targeting activity of this motif. In order to test this, I am performing site-directed mutagenesis on speicific amino acids within the α-helix to generate mutations that result in the destruction of the amphipathic property of the α-helix. I will then assay the impact of these mutations upon the

localization of Trm1-II. After refining the description of the important properties of this motif, future work will include searching other INM proteins for similar domains and testing whether mutations of these domains cause mislocalization of the proteins. This will help provide important information regarding the targeting mechanism of peripherally associated nuclear membrane proteins.

Chapter 1: Introduction

Eukaryotic cells have a membrane-bound nucleus that is essential for the life of the cell (Fig. 1). The nucleus is enclosed by the nuclear envelope, which is made up of an inner nuclear membrane (INM) and an outer nuclear membrane. Both the inner nuclear membrane and outer nuclear membrane are comprised of diverse groups of proteins. For example, nuclear pore complexes are made up of both integral membrane proteins that are located in the nuclear membrane and peripherally associated proteins that are located on the nuclear membrane. Another important subset of proteins specifically localizes to the INM, and these proteins are important for several functions. In fact, these INM proteins have been shown to play many vital roles in nuclear function, including chromatin organization, gene expression, and DNA metabolism (Hetzer, 2010). Specifically, INM proteins have been recently found to function as "transcription factor magnets' by sequestering transcription factors to the INM, which reduces the transactivation and transrepression activites of the factors" (Heessen, 2007).

In order for the inner nuclear membrane proteins to perform their vital roles in nuclear function, it is critical for them to achieve their proper subcellular destination. Failure to locate properly may lead to the loss of function, which can subsequently lead to disease. For example, greater than sixty genetic disorders, known as laminopathies, are caused by failure to correctly

localize proteins to the INM. Some examples of these disorders are cardiac and skeletal myopathies, partial lipodystrophy, peripheral neuropathy, and severe premature aging (Worman and Boone, 2007; Parnaik, 2008).

The INM contains two types of proteins, integral membrane proteins, which contain a transmembrane domain, and peripheral membrane proteins. In order to understand the genetic disorders that are caused by the mislocalization of INM proteins, it is important to learn how these proteins achieve their correct subcellular location. While much is still unknown, models have been proposed for the mechanism by which integral membrane proteins target to the INM. It is thought that integral membrane proteins that are synthesized on and anchored to the endoplasmic reticulum (ER) are directed from the ER/outer nuclear membrane to the INM via nuclear pore lateral channels (Soullam and Worman, 1995). Important studies in yeast have demonstrated that Heh1/Heh2 integral, INM proteins are directed from the ER to nuclear pores via a process requiring the Ran pathway, the nuclear localization signals (NLS) in Heh1/Heh2 proteins, and the karyopherins, importin-α and importin-β, that interact with these NLSs (King *et al*. , 2006). While some information regarding the targeting of integral membrane proteins to the INM is known, very little has been discovered about the targeting of peripherally associated INM proteins to this location. Since there are hundreds of peripherally associated INM proteins, many of which are very important for nuclear structure and organization, it is important for researchers to gain insight into the mechanism by which these proteins achieve the correct subcellular destination.

The budding yeast, *Saccharomyces cerevisiae*, is an extremely useful model organism to study the nucleus and nuclear membrane. One important property of *S. cerevisiae* that makes it a strong model for such studies is the fact that it undergoes a closed mitosis. During a closed

mitosis, the chromosomes divide within an intact nuclear membrane. Higher eukaryotes undergo an open mitosis during which the nuclear membrane breaks down before the chromosomes separate and then the membrane reassembles. A plethora of tools have been developed for the study of cell biology and genetics in yeast. Also, *Saccharomyces cerevisiae* has a sequenced genome, is easy to maintain, and is readily available. Most importantly, there is evidence suggesting that similar mechanisms for nuclear organization are shared between yeast and higher eukaryotes. For example, nuclear lamins, which are found in higher eukaryotes but not yeast, localized to the nuclear periphery when expressed in yeast, suggesting the possibility of conservation in mechanism (Smith and Blobel, 1994). The mislocalization of lamins in higher eukaryotes is responsible for the genetic disorders known as laminopathies. Discoveries in yeast regarding INM protein localization, therefore, have the potential to provide information that is to relevant these diseases.

Trm1, a tRNA methyltransferase, has been used as a reporter to study INM targeting of peripheral membrane proteins. In wild-type cells, two forms of the Trm1 protein are generated by alternative translation starts (Ellis *et al*., 1987). The longer form of the protein, Trm1-I, appears to be exclusively located in the mitochondria. Trm1-II, the shorter form of the protein, is located both in nuclei and in mitochondria, but it is predominantly located in the nucleus. Trm1-II is evenly distributed on the INM in wild-type cells. Several lines of evidence have demonstrated Trm1-II is peripherally associated to the INM (Rose *et al*., 1992; Murthi and Hopper, 2005). For example, Trm1 does not have a transmembrane domain, and it is released from the INM by reagents that release peripheral proteins.

Recent research has demonstrated that Trm1 contains a cis-acting motif that is necessary and sufficient for its targeting to the INM (Lai *et al*., 2009). Trm1 amino acids 130-151 have

been shown to make up this motif. When this region is deleted from the wild-type Trm1, the protein mislocalizes to the nucleoplasm instead of targeting to the INM. These data show that Trm1 amino acids 130-151 are necessary for INM targeting. When this amino acid region is fused with three other reporter proteins (β-galactosidase, GFP, or Trm7-GFP) the proteins target to the INM instead of the nucleoplasm. These data show that Trm1 amino acids 130-151 are also sufficient for INM targeting. When specific amino acids within this 130-151 region were randomly mutated, some of the mutations caused protein mislocalization to the nucleoplasm (Fig. 2; see amino acid changes above the Trm1 wild-type protein sequence). This supports the idea that the Trm1 amino acids 130-151 may be a targeting/tethering motif that is responsible for the targeting of Trm1 to the inner nuclear membrane.

These and other data provide a three-step model for how Trm1 localizes to the INM (Fig. 3). The first step of this model is entry into the nucleoplasm. The second step is the targeting of Trm1 to a specific spot on the INM. The third and final step is the spreading of Trm1 around the INM. The Trm1 peptide INM targeting motif likely functions in step two or three of this mechanism. The Trm1-II (130-151) region was the first peptide discovered to have INM targeting activity.

A targeting peptide motif can be either amino acid sequence specific or structure specific. If a motif is sequence specific, then certain amino acids are required at a specific location within the motif in order for it to function properly. Some excellent examples of amino acid specific motifs are nuclear localization sequences, which are often sequences that are rich in positively charged amino acids. Conversely, if a motif is structure specific, then some aspect of the protein structure that is formed by the motif is important for it to function properly. An example of a structure specific motif is the mitochondrial targeting sequence (MTS), which forms an

amphipathic α-helix with positively charged residues on one side and uncharged hydrophobic residues on the opposite side (Pfanner, 2000).

The overall goal of this research study is to determine how peripherally associated proteins target to the INM. Other proteins in yeast, as well as higher eukaryotes, may employ a similar mechanism or may have a similar motif that is responsible for their INM targeting. If this is the case, learning which properties of the Trm1 motif are important for INM targeting will potentially have a significant impact on the understanding of the targeting of other proteins to the INM. In an attempt to further characterize the Trm1-II targeting/tethering motif, a random mutagenesis screen was carried out, in which greater than sixty mutations causing various amino acid changes were created within the 130-151 region. The localization of Trm1, tagged with green fluorescent protein (GFP), was observed using fluorescence microscopy. Results of the screen were analyzed to determine which amino acids within the 130-151 region are important for the INM targeting function of the motif. Moreover, the question of whether the motif is sequence specific or structure specific was addressed.

Figure 1

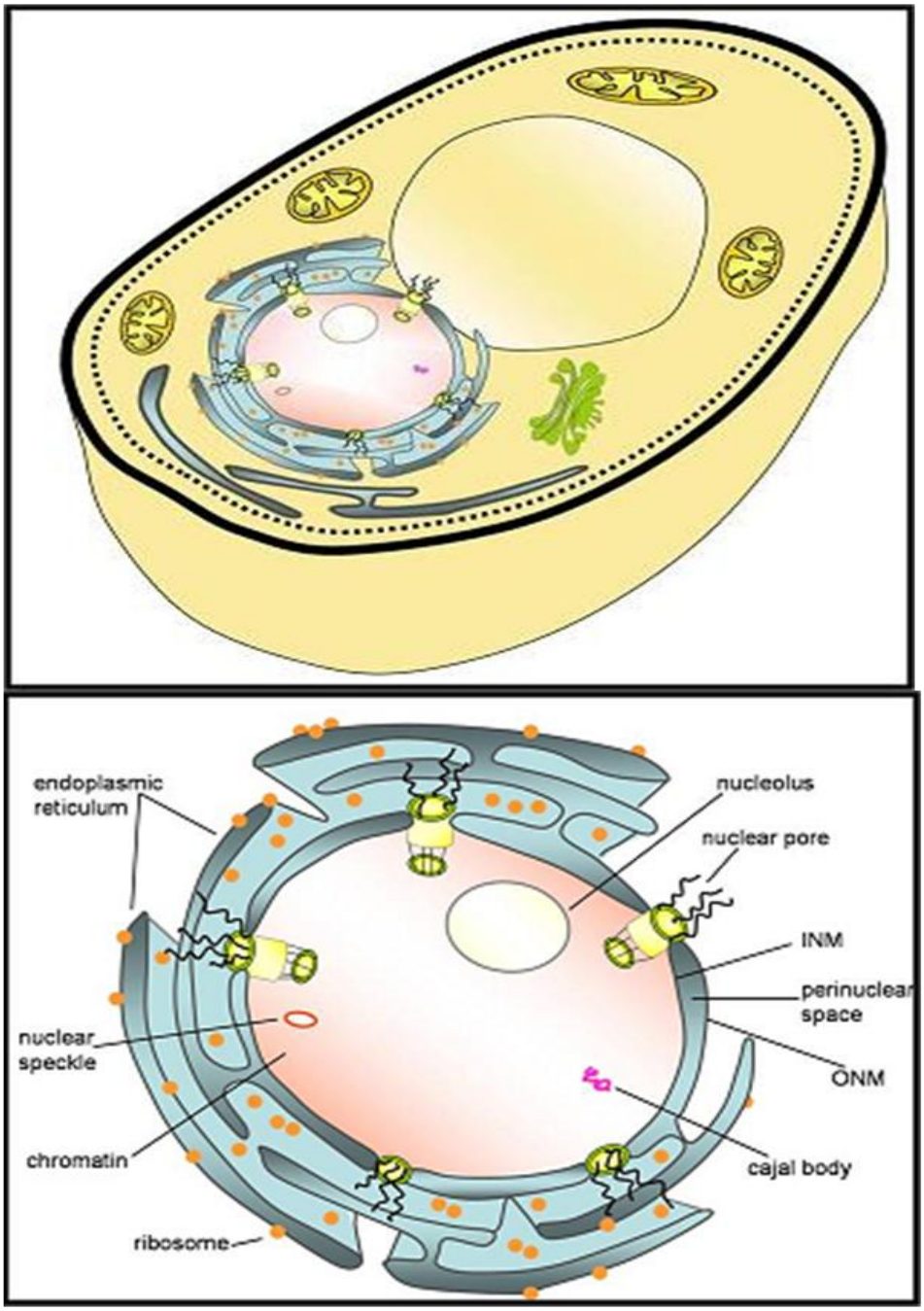

Figure 1: Top: Schematic representation of the yeast cell; bottom: schematic representation of the nucleus. (Greetchen Diaz with permission)

Figure 2

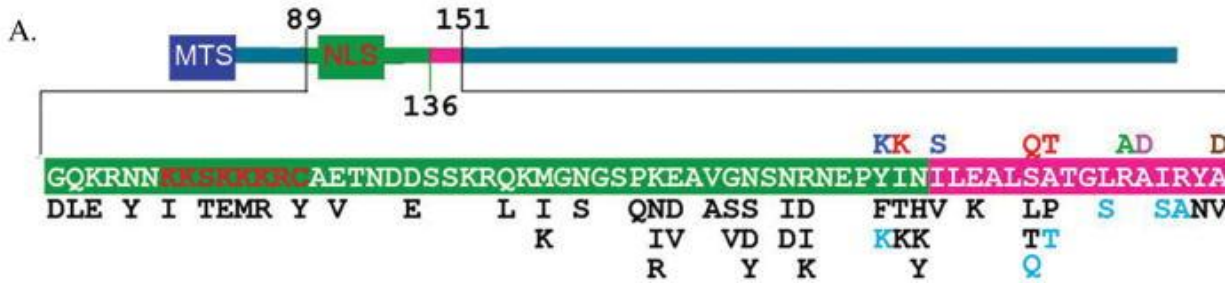

Figure 2: Green box indicates the area of Trm1 in eukaryotic but not Archael counterparts; Magenta box indicates sequences highly conserved in all Trm1 proteins. Mutations that alter the wild-type localization of Trm1 are listed above the wild-type sequence. Those changes that have no effect upon Trm1 localization are indicated below the sequence.

Figure 3

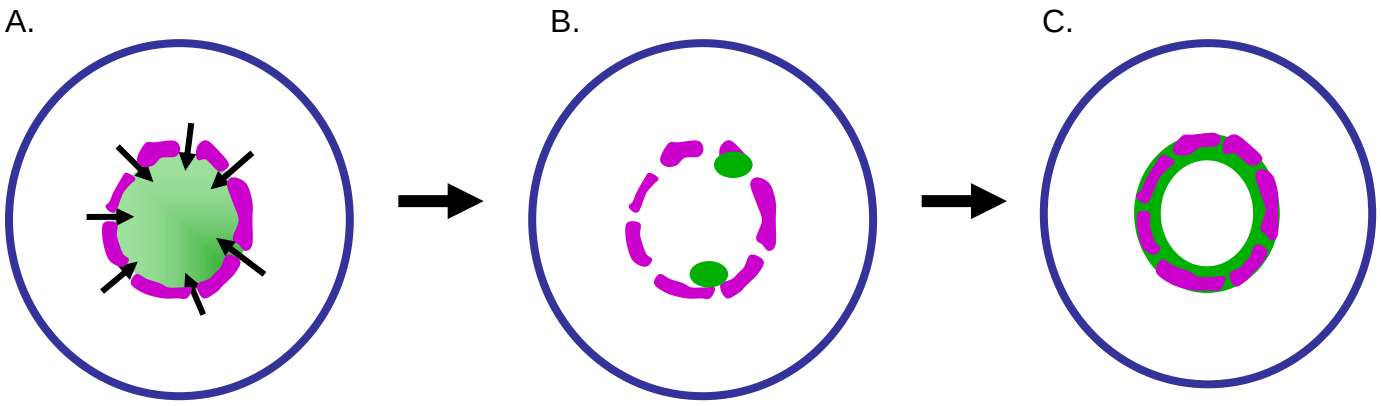

Figure 3: The 3-step model for how Trm1 targets to the INM. A. NLS-mediated entry into the nucleoplasm. B. Trm1 targets to a spot on the INM. C. Trm1 spreads around the INM. (Purple- INM; Green- Trm1-II-GFP)

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Table 1- Oligonucleotides used for site-directed mutagenesis

Name	**Sequence (5'-3')**	**Source**
JP001/TPL117 (Trm1-148+)	5'- ATTAGGTATGCTCATGAAATTCCC – 3'	This study
JP002/TPL118 (R146S)	5'- GGCTGATAACCCAGTGGCTGA- 3'	This study
JP003/TPL119 (R146E)	5' – GGCTTCTAACCCAGTGGCTGA – 3'	This study
JP004/TPL120 (R146W)	5'- GGCCCATAACCCAGTGGCTGA-3'	This study
JP005/TPL121 (A147R)	5'- GCGTCTTAACCCAGTGGCTGA – 3'	This study
JP006/TPL122 (A147W)	5' – CCATCTTAACCCAGTGGCTGA – 3'	This study
JP007/TPL123 (Trm1-152+)	5'- CATGAAATTCCCCATGTGAGGGAA-3'	This study
JP008/TPL124 (R149S)	5' – AGCATACGAAATGGCTCTTAA- 3'	This study
JP009/TPL125 (R149E)	5'- AGCATACTCAATGGCTCTTAA- 3'	This study
JP010/TPL126 (A151W)	5'- CCAATACCTAATGGCTCTTAA-3'	This study
JP011/TPL127 (A151K)	5-TTTATACCTAATGGCTCTTAA – 3'	This study
T7	5'-TAATACGACTCACTATAGGG-3'	pGEM kit, Promega
SP6	5'-TATTTAGGTGACACTATAG-3'	pGEM kit, Promega

Chapter 2: Materials and Methods

Strains and Media

BY4741 (MAT-α) was used for all yeast experiments. Cells were grown in yeast extract peptone dextrose media (YEPD). After yeast transformation, strains were grown at 30°C for 2 days on defined media lacking uracil. For galactose induction, cells were grown for 2 days at 30°C on media lacking uracil and containing raffanose as the carbon source. Transcription of Trm1 is regulated by the GAL-promoter which was induced for two hours using 1/10 volume of 20% galactose. *E. coli* cells were grown in LB media with ampicilin or on LB+Amp plates at 37°C.

Plasmids

-pGP54a-Trm1-II-GFP

-pGEMt

Random Mutagenesis

Error prone mutagenesis was performed using a GeneMorph® II EZClone Domain Mutagenesis Kit (Stratagene) to generate random mutations of Trm1 amino acids 126-151. Error prone PCR was performed under the following conditions: 1 cycle, 2 min. at 95°C; 30 cycles, 30 sec at 95°C, 30 sec at 60°C, 1 min at 72°C; 1 cycle, 10 min at 72°C using pGP54a-Trm1-II-GFP as the template. This created a library containing 1-5 nucleotide mutations in the region encoding amino acids 130-151, which was subsequently used as a mega primer to introduce the mutations into wild-type *TRM1* in plasmid pGP54a-Trm1-II-GFP following the instructions provided by Stratagene. The PCR product was then digested with *Dpn*1 to destroy the nonmutated donor plasmid DNA. The plasmid library was then transformed into *E. coli* (as described below). (See Fig 4 for method)

Plasmid DNA Purification

Qiagen's QIAprep Miniprep kit was used for all plasmid DNA purification. Liquid cultures of *E. coli* cells containing the plasmid were grown overnight at 37°C. The bacteria cells were first lysed under alkaline conditions. The lysate was subsequently neutralized and adjusted to high-salt binding conditions. The plasmid was then adsorbed in a QIAprep column to a silica membrane in a high-salt buffer. Endonucleases were then removed with a brief wash step. A second wash step

with a buffer containing ethanol was used to remove salt. The plasmid DNA was then eluted from the QIAprep column.

E. coli Transformation

Plasmids were added to chemical competent cells. The cells were kept on ice for 30 minutes and then heat shocked at 42° C for 45 seconds. Cells were subsequently put on ice for one minute for recovery. The cells were then grown in nutrient rich SOC media at 37°C for one hour. Finally, the cells were plated on YT+Amp media for plasmid selection (as the plasmids were Amp-resistant) and grown overnight at 37°C.

Yeast Transformation

Wild-type yeast was grown for 2 days in liquid YEPD media at 23° C. A buffer, including 60% polyethyleneglycol, 1M lithium acetate, 1M dithiothreitol, and sheared salmon sperm single stranded DNA, was added to the wild-type yeast cells. The plasmid was subsequently added. Cells were placed in a 45° C water bath for 30 minutes. Cells were then plated on media lacking uracil and grown at 30° C for 2 days.

Fluorescence Microscopy

Fluorescence microscopy and image capture was done with Nikon90i equipped with a Cool-SNAP HQ2 digital camera and MetaMorph software (Molecular Devices, Sunnyvale, CA). Image processing was performed using Adobe photoshop.

Sequencing

Plasmids were sent to the Plant-Microbe Genomics Facility at The Ohio State University for sequencing. Sequences were then compared to the wild-type *TRM1* sequence using BLAST to define the mutations generated.

Structural Predictions

We employed the open source PyMol 3-D imaging program to superimpose the generated amino acid mutations on the 3-D crystal structure of the Archea Trm1 (10).

Site-directed Mutagenesis

Two fragments were generated by PCR amplification using pGEM-T/Trm1-II as the template and *Pfu* Ultra High Fidelity DNA polymerase (Stratagene). The first fragment was generated using the T7 primer and the Trm1 primer which contained one of several mutations (see Table 1). The second fragment was generated using an SP6 primer and the appropriate downstream primer for each construct. The purified PCR products were then digested with *Eco*RI and *Hind*III to remove them from the pGEM-T vector. The fragments were then treated with PNK and ligated into a pGP-54a-GFP plasmid that was previously digested with *Eco*RI and *Hind*III. (See Fig. 5 for method)

Figure 4:

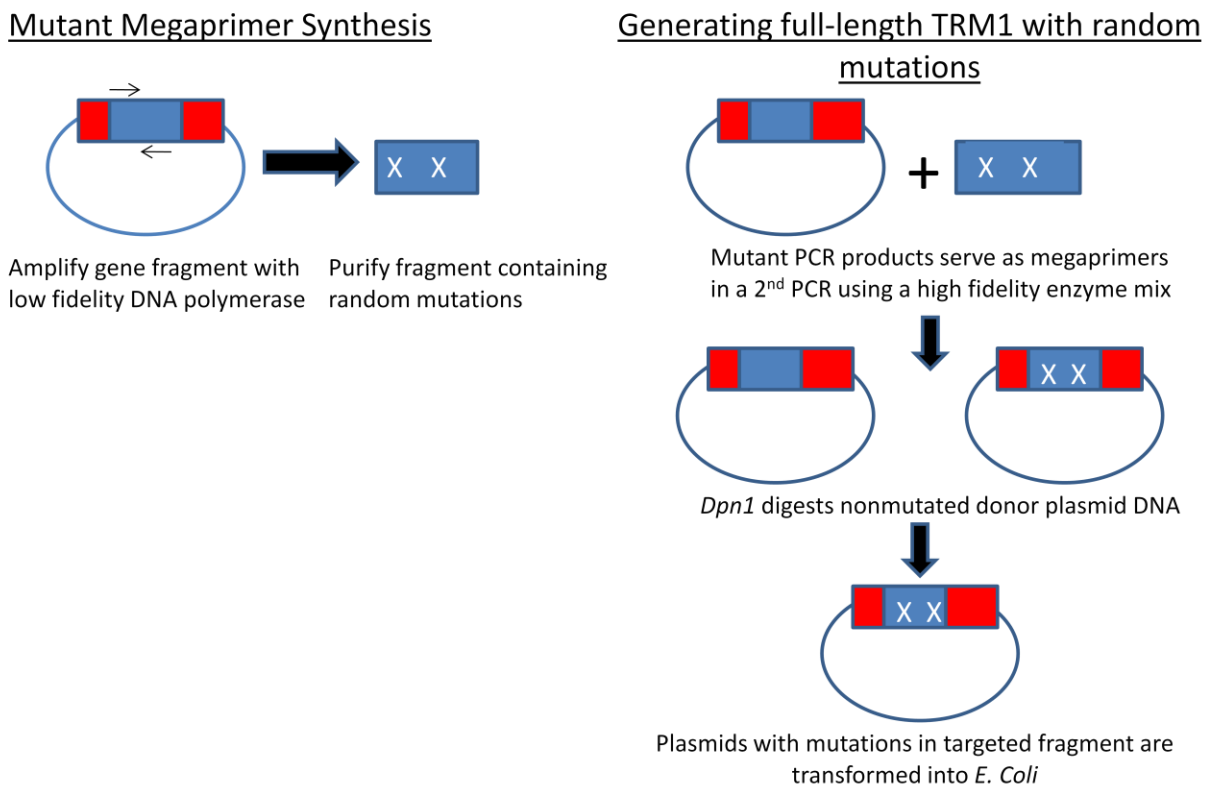


Figure 4: Method used for random mutagenesis of Trm1(126-151). A.) Generate mutant megaprimer. B.) Use mutant megaprimer to generate full length TRM1 with random mutations.

Figure 5

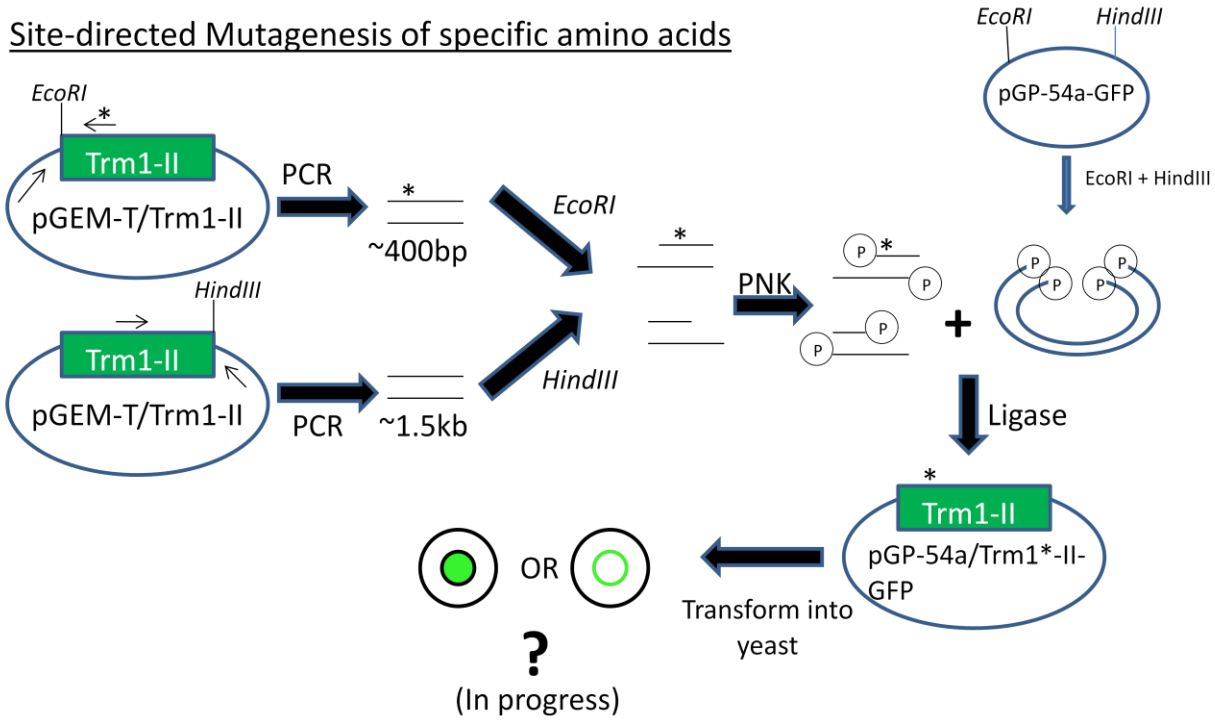


Figure 5: Method used for site-directed mutagenesis.

Chapter 3: Results

Random Mutagenesis Screen

A random mutagenesis screen was performed in an attempt to further characterize the Trm1 (130-151) targeting/tethering motif. The Trm1 amino acid region 126-151 was targeted in the random mutagenesis. A library which contained approximately three hundred and sixty clones, each presumably containing a random mutation(s) within the Trm1 (126-151) sequence, were generated. The plasmid DNA was purified and subsequently transformed into yeast.

Fluorescence microscopy was then used to observe the localization of Trm1-GFP protein. The wild-type Trm1 protein locates to the periphery of the INM. If a mutation caused a defect in the INM targeting/tethering activity of the motif, then the protein would be mislocalized to the nucleoplasm. The intention was to determine which amino acid changes do or do not cause a defect in the targeting/tethering activity. Approximately one hundred and sixty of the generated plasmids were then sequenced, and each sequence was compared to that of wild-type Trm1. Each discrepancy between the plasmid sequence and wild-type sequence was analyzed in an attempt to determine if an amino acid change occurred. Many clones contained plasmids with a single amino acid mutation, while some contained plasmids with double or triple amino acid mutations. The plasmids from about one hundred forty different mutants that showed wild-type localization of Trm1 were sequenced. Approximately fifty of these plasmids contained an amino acid change, and about thirty-five of these changes were new mutations that result in the wild-type localization of Trm1. Approximately fifteen plasmids from mutants in which Trm1 was mislocalized to the nucleoplasm were sequenced. From these sequences, thirteen new amino acid changes were found that cause a defect in the targeting/tethering activity of the Trm1(130-151) motif. Every amino acid within the targeted region was mutated, and several of the mutations generated within this region cause mislocalization of Trm1 (Fig. 6- lists all amino acid changes generated). These data confirm that this specific region is important for INM targeting.

The Trm1 (130-151) motif appears to be structure specific

The data indicated that the Trm1-II (130-151) region is likely a structural motif. The results of the random mutagenesis screen do not indicate that it is necessary for a specific amino acid to be present at any of the locations within the motif, so it is unlikely that this is a sequence

specific motif. For example, amino acid 148 is an isoleucine in the wild-type Trm1 amino acid sequence, and this residue can be changed to serine, asparagine, or threonine and the motif does not lose its targeting/tethering activity. This indicates that the presence of an isoleucine is not necessary at that location in the sequence. Examples such as amino acid 148 support the idea that this motif is not sequence specific. Instead, it appears that the properties of the amino acids, such as size, hydrophobicity, and charge, are important.

In order to determine whether or not changes in amino acid properties were caused by each of the various mutations, I evaluated and categorized each mutation and naturally occurring amino acid by its property (Fig. 7). Each amino acid was categorized as either hydrophobic (AGCVPLIM), hydrophilic-neutral (NQST), positively charged (RKH), negatively charged (ED), or bulky/aromatic (WFY). The data generated by the random mutagenesis indicate that almost all of the mutations that cause a defect in the Trm1 targeting/tethering motif also create a change in the property of the wild-type amino acid. For example, when the property of amino acid 151 is changed from hydrophobic to negatively charged, Trm1 is mislocalized. However, when this same amino acid is changed to another hydrophobic amino acid, Trm1 localizes to the periphery of the INM as if it were wild-type. This supports the idea that the properties of the amino acids are important for the maintenance of the targeting/tethering activity of the motif. It is likely the properties of the amino acids that are important, therefore, it is probable that this is a structure specific peptide motif.

If the data had indicated that this was a sequence specific motif, a possible consensus sequence that may have been conserved among other proteins in yeast and in higher eukaryotes could have been determined. If a similar sequence existed in other proteins that target to the INM, it could have been determined that this sequence specific motif is responsible for INM

targeting of peripherally associated proteins. Since the data instead indicated that the motif is structure specific, it was necessary to conduct further investigation of the motif to determine which properties of the amino acids within the sequence are responsible for maintaining its structure.

Structural Predictions

An amphipathic α-helix appears to be important for INM targeting activity

It was necessary to determine which aspects of the Trm1 structure may be important for the INM targeting activity of the 130-151 motif. First, the generated amino acid mutations were superimposed on the recently defined Archea structure of Trm1 (Ihsanawati *et al*., 2008) in an attempt to determine the affect of the mutations on the structure of the protein. The region that is homologous to TrmI-II (130-151) in yeast was analyzed. The overlay indicated that amino acids 145-153 make up an α-helix. The helix is part of the motif region that is necessary and sufficient for the INM targeting of Trm1-II. Several of the mutations that cause Trm1 mislocalization can be found in the region that makes up the α- helix, so it is probable that this component of the Trm1 structure is important for its targeting to the periphery of the INM. Since this α-helix appeared to be important, further analysis of the 145-153 region was conducted. When analyzing the amino acids that make up this helix, it was realized that this helix is amphipathic. A helical wheel was used to visualize the composition of the helix formed by amino acids 145-153 (Fig. 8). The helical wheel indicates that this helix has four polar amino acids (R146, R149, E153, and Y150) on one side and three nonpolar amino acids (A147, A151, and I148) on the other side. The next step was to determine whether or not the amino acid changes within the helical region that cause Trm1 mislocalization result in a change in amino acid property. Analysis revealed that all

of the mutations within the 145-151 amino acid region resulted in a deviation from the property of the wild-type amino acid. Moreover, the data indicate that amino acid changes that altered the amphipathic property of the α-helix resulted in the mislocalization of Trm1-II to the nuceloplasm. For example, a mutation that changed the polar amino acid arginine (residue 146) to the nonpolar amino acid serine caused mislocalization. Conversely, a mutation that changed the nonpolar amino acid alanine (residue 147) to the negatively charged polar amino acid aspartate also caused mislocalization of Trm1 to the nucleoplasm. Since the destruction of the amphipathic property of the helix appears to cause a defect in the targeting/tethering motif , a model in which the amphpathic property of the α-helix is important for INM targeting/tethering activity was proposed.

Bulky residues may interfere with Trm1(130-151) targeting activity

Based on the results of the random mutagenesis screen, we have also determined that amino acid changes resulting in a bulky or aromatic amino acid likely destroy the INM localization of Trm1-II. For example, a mutation that changed the small amino acid isoleucine (residue 148) to the bulky amino acid phenyalanine caused Trm1 to mislocalize to the nucleoplasm. These data suggest that the size of each individual amino acid is also important for Trm1-II targeting to the INM.

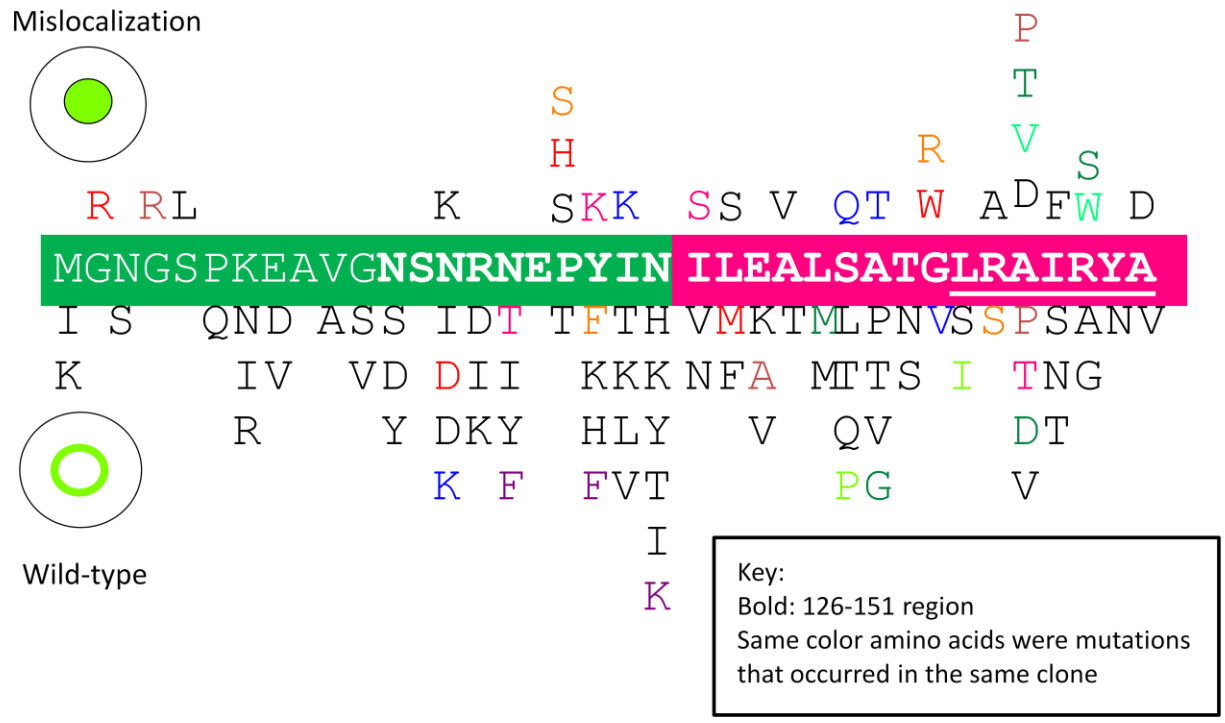
Site-directed Mutagenesis

In an attempt to test the model that was generated based on the results of the random mutagenesis screen, I am currently performing site-directed mutagenesis on specific amino acid residues that are part of the α-helix. The goal is to generate various mutations that will cause amino acid changes that will result in the destruction of the amphipathic property of the α-helix. For example, one of the site-directed mutations will cause the nonpolar amino acid alanine (residue 147) to change to the polar residue lysine. Another site-directed mutation will change the polar, positively charged amino acid arginine (residue 145) to the nonpolar amino acid serine. In addition to creating mutations that destroy the amphipathicity of the α-helix, mutations will also be made that cause small amino acids to be changed to bulky amino acids (such as phenylalanine and tryptophan). For example, one of the site-directed mutations will change the small amino acid alanine (residue 147) to the bulky amino acid tryptophan.

I have designed primers that will create nine different amino changes within the helical region (refer to table 1 for primers). Each individual mutation is being generated using the technique described in materials and methods for site-directed mutagenesis. Once plasmids containing the specific mutations are created and transformed into yeast, the localization of Trm1-GFP will be observed using fluorescence microscopy. The effect of the various mutations on the localization of Trm1-II to the INM will be determined. If mutations that alter the amphipathic property of the Trm1(145-153) α- helix cause mislocalization to the nucleoplasm, then there will be evidence that the amphipathic property is important for the targeting activity of Trm1-II. If bulky residues cause the mislocalization of Trm1-II to the nucleoplasm, then there will be evidence that the size of the amino acids is also important for INM targeting.

Figure 6

A.


Mislocalization
Wild-type
MGNGSPKEAVGNSNRNEPYINILEALSATGLRAIRYA
Key:
Bold: 126-151 region
Same color amino acids were mutations that occurred in the same clone

B.

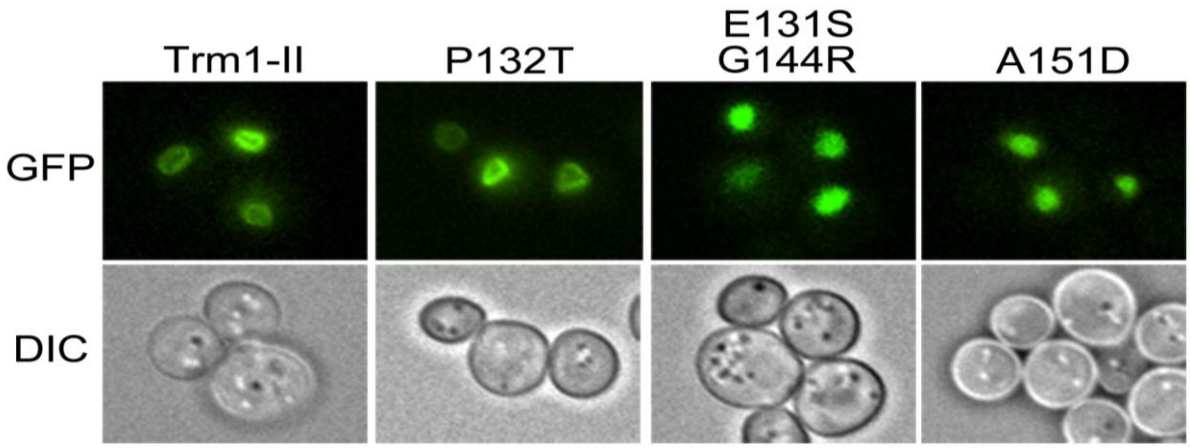


Figure 6: Mutations that do or do not alter Trm1 INM localization. A. A diagram of the mutations analyzed. Green box indicates the area of Trm1 in eukaryotic but not Archael counterparts; Magenta box indicates sequences highly conserved in all Trm1 proteins. The area of Trm1 that was subject to a random mutagenesis is indicated in bold. The underlined region indicates the amino acids that make up the amphipathic α-helix. Mutations that alter the location of Trm1-II GFP are shown above the wild-type sequence, and those that do not alter the location are shown below. (Black-single mutations ; color coordinated multiple mutations) B. Sample data for the random mutagenesis mutations: 1, wild-type Trm1-II- GFP; 2, mutation of amino acid (P132T) does not affect Trm1-II-GFP subnuclear location; 3 and 4 single (A151D) and double mutations (P131S, G144R) cause Trm1-II-GFP to mislocalize to the nucleoplasm.

Figure 7

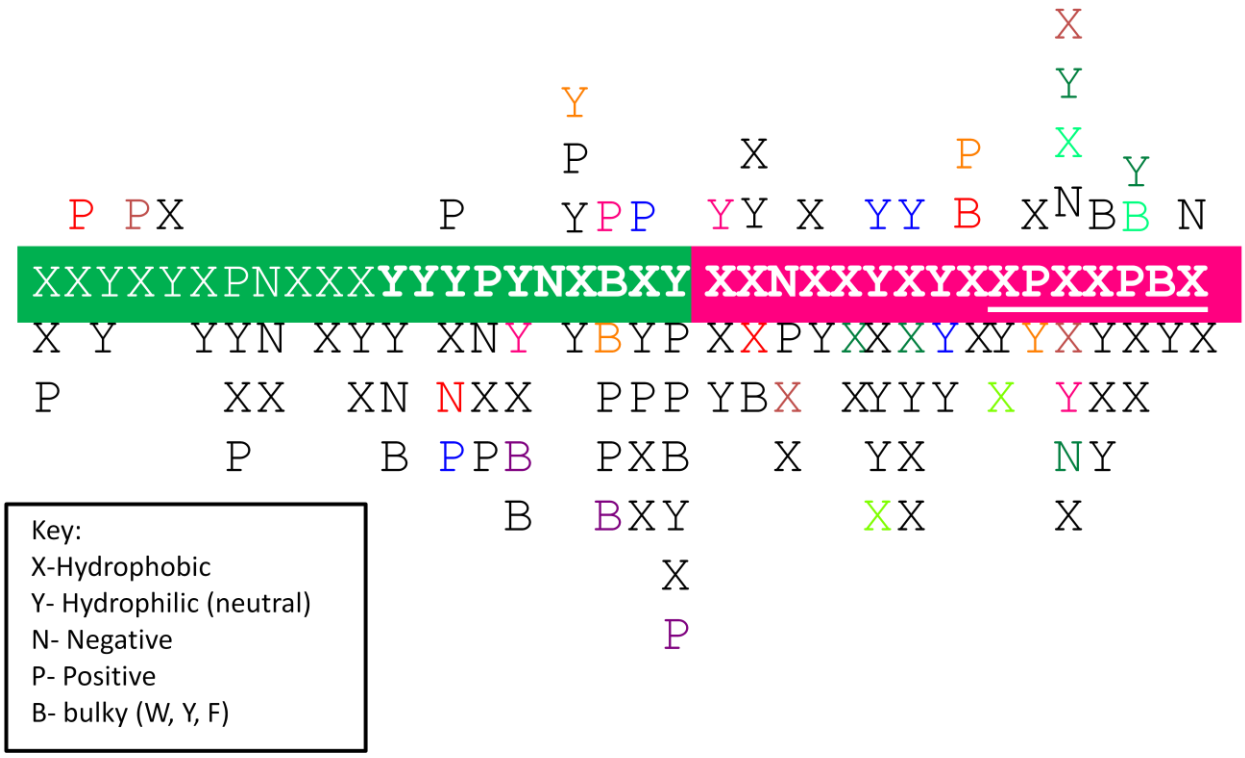
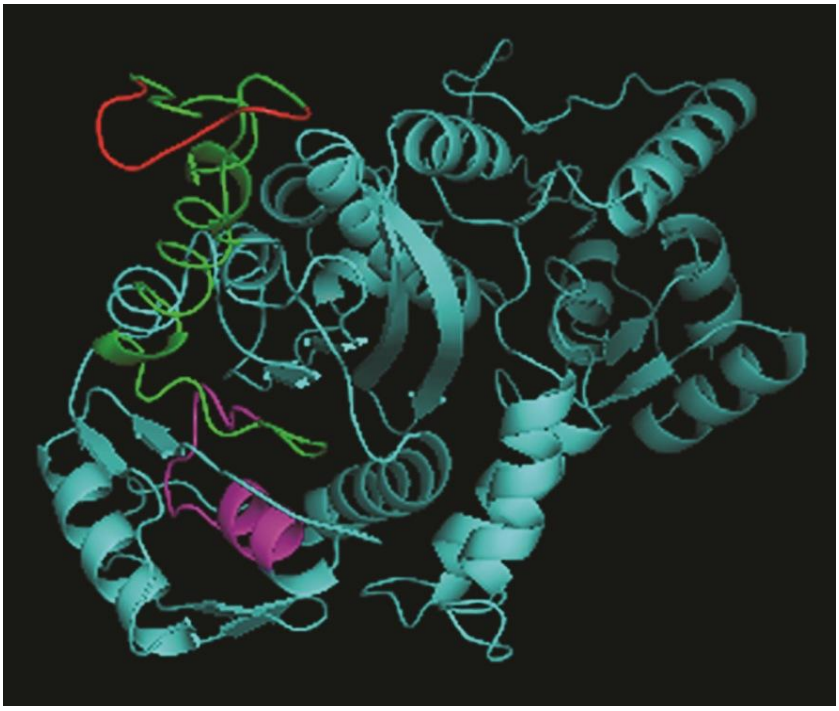


Figure 7: Changes in amino acid properties: Amino acids are listed by their property as indicated in the key. All other aspects of the figure remain the same as Figure 4A.

Figure 8

A.



B.

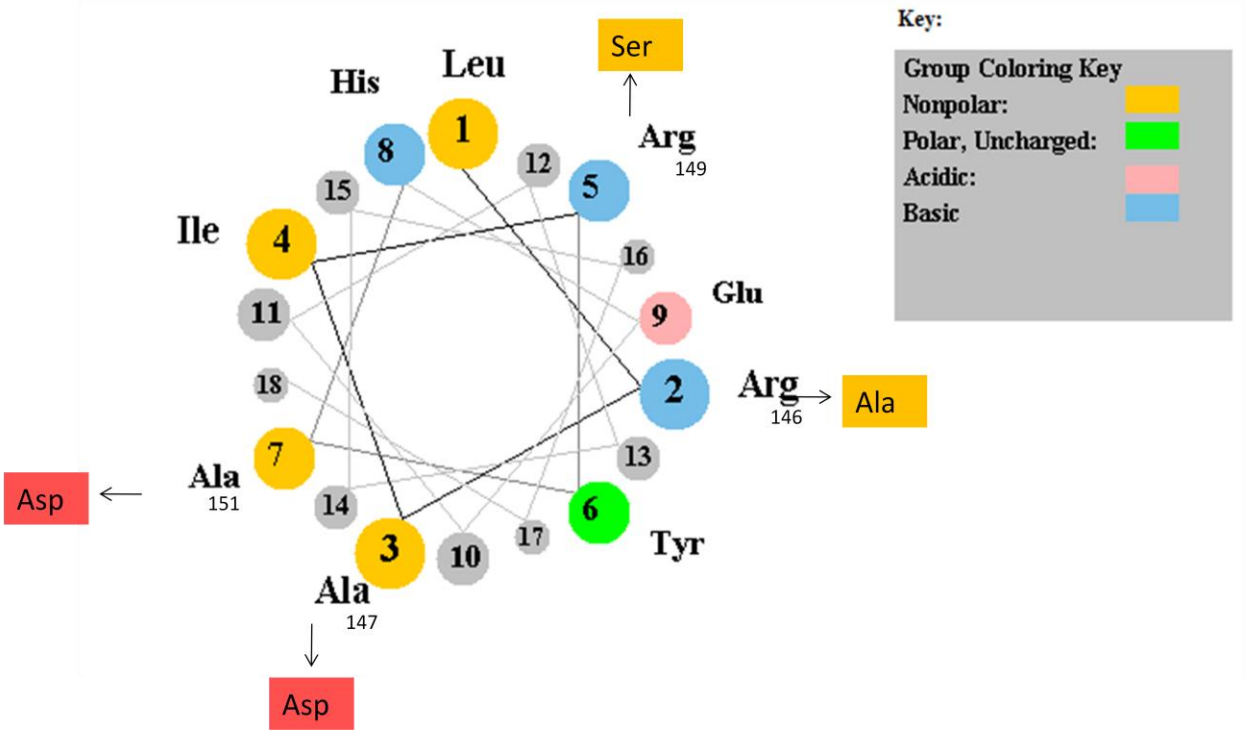

Key:
Group Coloring Key
Nonpolar:
Polar, Uncharged:
Acidic:
Basic
Ser
Leu
His
Arg
149
Ile
Glu
Arg
146
Ala
Asp
Ala
151
Tyr
Ala
147
Asp
1
2
3
4
5
6
7
8
9
10
11
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Figure 8: A. Ribbon structure of Archea Trm1. The helix generated by Trm1 amino acids 145-153 is indicated in purple. B. Helical wheel demonstrating the amphipathic property of the helix. (1=amino acid 145). The right side of the helix is composed of polar residues and the left side is composed of nonpolar residues. Some amino acid changes within the helix that cause Trm1 to mislocalize to the nucleoplasm are indicated.

Chapter 4: Discussion

The proper subcellular localization of proteins is often essential to their function. While there is some understanding about the mechanism by which integral membrane proteins target to the inner nuclear membrane, very little is known about how peripherally associated proteins target to the INM. Recent research in *Saccharomyces cerevisiae* has demonstrated that Trm1, a tRNA methyltransferase, has a cis-acting peptide motif, Trm1(130-151), that is necessary and sufficient for its targeting to the INM. This research was a starting point for understanding a mechanism by which peripherally associated proteins target to and are retained at the inner nuclear membrane. In the work reported here, the Trm1 targeting/tethering motif was further characterized, and a model has been proposed where the amphipathic property of the α-helix that is part of the motif is important for INM targeting. It is possible that other INM proteins in yeast, and higher eukaryotes, may contain a similar motif. If this is the case, it can be determined if this model is generally applicable to other proteins that target to the periphery of the INM.

In the work reported here, the Trm1(130-151) peptide motif was subjected to a random mutagenesis screen. About thirteen new mutations that cause Trm1 to mislocalize to the nucleoplasm and approximately thirty-five mutations that allow Trm1 to localize to the INM as if it were wild-type were characterized. Analysis of the results of the random mutagenesis screen indicated that a sequence specific motif is unlikely. The structure of the motif, especially the amphipathic helix, appears to be important for proper INM localization.

Further investigation of the effects of the various mutations on the structure of the Trm1 targeting/tethering motif was carried out. The structural analysis revealed that Trm1 amino acids 145-153 are predicted to form an amphipathic α-helix. The helix is part of the region that has been shown to be

necessary and sufficient for the INM targeting of Trm1. Mutations that destroyed the amphipathic property of the helix caused Trm1 to mislocalize to the nucleoplasm. Based on these data, I propose that the amphipathic property of the Trm1(145-153) α-helix is important for the targeting activity of the motif. In addition to the importance of the amphipathic property, the data also suggests that the size of the amino acid acids may be important for the peptide motif's targeting activity.

In order to test the proposed model in which the amphipathic property of the α-helix is important for the targeting/tethering activity of the Trm1(130-151) motif, site-directed mutagenesis is currently being performed. This experiment aims to generate a series of mutations that destroy the amphipathic properties of the helix. If the destruction of this property causes mislocalization of Trm1-II, it would suggest that the amphipathic property of the helix is important for the proper targeting of Trm1-II to the INM. A second set of mutations will change the size of some specific amino acids from small to bulky residues. This will test the idea that the size of the amino acids is also important for INM targeting.

No other motifs have been proposed for the targeting of peripherally associated proteins to the INM. The Trm1(130-151) cis-acting peptide motif is the first of its kind to be discovered. It is proposed here that the amphipathic α-helix is a component of this motif that has significant importance for its targeting activity. It is possible that other peripherally associated INM proteins contain a similar region. In the future, genome databases can be used to search for similar amphipathic α-helices in other peripherally associated INM proteins in yeast and in higher eukaryotes. Such proteins can then be studied to test whether or not the identified amphipatic α-helices function in INM targeting in the relevant proteins and in reporter proteins. If similar function structures are found in other proteins, then it can be concluded that the importance of the amphipathic helix in the targeting motif is not Trm1 specific and that we have uncovered a general mechanism for tethering/targeting of proteins to this important subnuclear location. The results obtained from this research have significantly contributed to the understanding of the targeting of peripherally associated proteins to the INM and may possibly have medical implications in the future.

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